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Inhibition of Proliferation of Estrogen Receptor–Negative MDA-MB-435 and –Positive MCF-7 Human Breast Cancer Cells by Palm Oil Tocotrienols and Tamoxifen, Alone and in Combination^{1,2}

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ABSTRACT Tocotrienols are a form of vitamin E, having an unsaturated isoprenoid side-chain rather than the saturated side-chain of tocopherols. The tocotrienol-rich fraction (TRF) from palm oil contains α -tocopherol and a mixture of α -, γ - and δ -tocotrienols. Earlier studies have shown that tocotrienols display anticancer activity. We previously reported that TRF, α -, γ - and δ -tocotrienols inhibited proliferation of estrogen receptor-negative MDA-MB-435 human breast cancer cells with 50% inhibitory concentrations (IC₅₀) of 180, 90, 30 and 90 μ g/mL, respectively, whereas α -tocopherol had no effect at concentrations up to 500 μ g/mL. Further experiments with estrogen receptor-positive MCF-7 cells showed that tocotrienols also inhibited their proliferation, as measured by [³H] thymidine incorporation. The IC₅₀s for TRF, α -tocopherol, α -, γ - and δ -tocotrienols were 4, 125, 6, 2 and 2 μ g/mL, respectively. Tamoxifen, a widely used synthetic antiestrogen inhibits the growth of MCF-7 cells with an IC₅₀ of 0.04 μ g/mL. We tested 1:1 combinations of TRF, α -tocopherol and the individual tocotrienols with tamoxifen in both cell lines. In the MDA-MB-435 cells, all of the combinations were found to be synergistic. In the MCF-7 cells, only 1:1 combinations of γ - or δ -tocotrienol with tamoxifen showed a synergistic inhibitory effect on the proliferative rate and growth of the cells. The inhibition by tocotrienols was not overcome by addition of excess estradiol to the medium. These results suggest that tocotrienols are effective inhibitors of both estrogen receptor-negative and -positive cells and that combinations with tamoxifen should be considered as a possible improvement in breast cancer therapy. *J. Nutr.* 127: 544S–548S, 1997.

KEY WORDS: • tocotrienols • tamoxifen • breast cancer • MCF-7 • MDA-MB-435

Experiments in other laboratories have shown that diets containing a high level of palm oil do not promote mammary carcinogenesis in rats (Kritchevsky et al. 1992, Sundram et al. 1989). Saturated fats, such as coconut oil and beef tallow, also failed to promote mammary carcinogenesis in rats (Carroll and Khor 1971) evidently because they contain insufficient polyunsaturated fatty acid (linoleic acid) (Ip 1987). However, this does not appear to be the limiting factor with palm oil.

Palm oil stripped of its vitamin E fraction promoted mammary carcinogenesis like other unsaturated fats and oils, and addition of the vitamin E fraction from palm oil to a high corn oil diet tended to counteract the promoting effect of the dietary corn oil (Nesaretnum et al. 1992). The vitamin E of palm oil, unlike that of most other fats and oils, consists largely

of tocotrienols, the remainder being α -tocopherol (Fig. 1) (Ong 1993, Sundram et al. 1989). This fraction of the oil, referred to as the tocotrienol-rich fraction (TRF)⁴ or Palm Vitee, was shown to inhibit the proliferation and growth of human breast cancer cells in vitro, whereas α -tocopherol was ineffective. The individual tocotrienols of TRF were also found to inhibit the cancer cells more effectively than would be expected from results obtained with the TRF itself (Carroll et al. 1995).

The above experiments were conducted with estrogen receptor–negative MDA-MB-435 cancer cells (Price et al. 1990). The ability of TRF and tocotrienols to inhibit MCF-7 estrogen receptor–positive cells (Soule et al. 1973) was subsequently investigated. TRF and the individual tocotrienols inhibited this line more effectively than α -tocopherol, but not as effectively as tamoxifen (Guthrie et al. 1995).

Tamoxifen, an estrogen antagonist, is used extensively in the hormonal therapy of breast cancer (Jordan 1994, Powles

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⁴ Abbreviations used: DCC, dextran-coated charcoal; DMSO, dimethyl sulfoxide; ER, estrogen receptor; FCS, fetal calf serum; IC₅₀, 50% inhibitory concentration; MTT, 3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide; OD, optical density; TRF, tocotrienol-rich fraction.

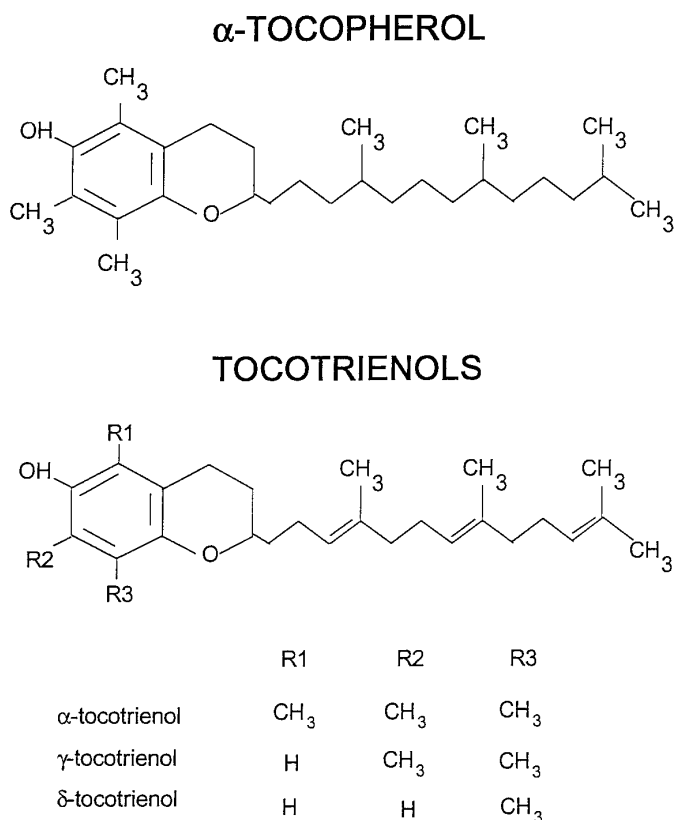


FIGURE 1 Structures of α -tocopherol and tocotrienols.

and Hickish 1995). Tamoxifen acts mainly by blocking the stimulatory action of estrogens in hormone-responsive breast cancer cells (Haran et al. 1994, Jordan 1995), but has dual dose-dependent estrogenic/antiestrogenic properties (Horwitz et al. 1978). The use of tamoxifen is limited, however, by the development of resistance to this compound in most patients (Osborne et al. 1995). Because many recurrent breast cancers are estrogen receptor-independent, a drug or dietary agent that inhibits the growth of both estrogen receptor-positive and -negative tumors would be of great interest (Peterson and Barnes 1991).

In this paper, we report the effects of TRF, α -tocopherol and the individual tocotrienols of palm oil on MCF-7 estrogen-responsive human breast cancer cells. The compounds were also tested in 1:1 combinations with tamoxifen on both MCF-7 and MDA-MB-435 cells.

MATERIALS AND METHODS

Materials. TRF, α -tocopherol and the individual tocotrienols were obtained from the Palm Oil Research Institute of Malaysia (PORIM), Kuala Lumpur. Tamoxifen and 17- β estradiol were purchased from Sigma Chemical (St. Louis, MO). MDA-MB-435 cells (Price et al. 1990) were obtained from Janet Price (M. D. Anderson Cancer Center, Houston, TX) and MCF-7 (Soule et al. 1973) cells were obtained from the American Tissue Culture Collection (Rockville, MD). Tissue culture medium and fetal calf serum were purchased from GIBCO (Burlington, ON). Fetal calf serum treated with dextran-coated charcoal (FCS/DCC) was purchased from Cocalico Biologicals (Reamstown, PA). Trypsin was obtained from Difco Laboratories (Detroit, MI) and [³H] thymidine (248 Bq/mmol) was purchased from ICN (Irvine, CA). All other chemicals were from Sigma.

Cell culture. MDA-MB-435 cells (estrogen receptor-negative human breast cancer cells) were maintained at 37°C in minimum essential medium containing 3.7g of NaHCO₃/L, supplemented with

10% (v/v) FCS. The medium was equilibrated with a humidified atmosphere of 5% CO₂. Stock cultures were seeded at a density of 2 × 10⁵ cells/mL and allowed to multiply for 48–72 h.

MCF-7 (estrogen receptor-positive human breast cancer cells) were maintained in minimum essential medium containing 3.7g NaHCO₃ supplemented with 10% FCS, 1 mmol/L sodium pyruvate, 10 μ g/mL insulin and 1% (v/v) fungizone (antibiotic/antimycotic). The cells were grown at 37°C in a humidified atmosphere containing 5% CO₂ and passaged weekly, using 0.25% trypsin.

Experimental media. Stock solutions of TRF, α -tocopherol, α -, γ -, and δ -tocotrienols were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/mL and then diluted into the culture medium so that the final concentration of DMSO was <0.1%. The same amount of DMSO was added to control cells. Tamoxifen was dissolved in ethanol at a concentration of 50 mg/mL and a similar control was conducted.

Incorporation of [³H] thymidine into DNA. MDA-MB-435 cells were plated at 2 × 10⁴ cells/well in 96-well, flat-bottomed culture plates in a total volume of 200 μ L of medium and incubated at 37°C for 48 h with or without test compounds. [³H] Thymidine (18.5 μ Bq/well) was then added; after 4 h, the cells were harvested onto a glass fiber filter paper using a semiautomatic 12-well cell harvester (Skatron, Sterling, VA). Radioactivity on the filter paper was counted, using Scintiverse in a liquid scintillation counter (Kothapalli et al. 1993). For the MCF-7 cells, 5 d prior to use, the growth medium was exchanged for phenol red-free medium containing 10% FCS that had been treated with dextran-coated charcoal (DCC). The cells were then trypsinized and 2 × 10⁴ cells/well were plated as described above. Two days later, the medium was replaced with an experimental one containing 2.5% FCS/DCC and the test compounds for 5 d (Thomas and Monet 1992). Untreated cells were used as a control. [³H] Thymidine was then added and the cells harvested as described above.

Growth experiment. MDA-MB-435 and MCF-7 cells were plated at 1 × 10⁴ and 5 × 10⁴ cells/dish, respectively, in 60-mm dishes with or without test compounds at their 50% inhibitory concentration (IC₅₀). The cells were removed by trypsinization at the specified times and counted, using a hemacytometer.

Viability of cells. Viability of cells was measured by an assay described by Hansen et al. (1989). In this assay, a tetrazolium salt, 3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide (MTT), is converted to a blue formazan product by dehydrogenases that are active in living cells. The intensity of the blue color that develops is a measure of cell viability. MDA-MB-435 and MCF-7 cells (8 × 10⁴) were seeded with various concentrations of the tocotrienols and/or tamoxifen in a 96-well flat-bottomed plate in a total volume of 200 μ L of medium. MTT (25 μ L of 5 mg/mL) was added to each well. After 3 h, 100 μ L of extraction buffer, consisting of 20% SDS, dissolved in a 50% DMF:50% water solution at pH 4.0, was added. The blue color formed was measured at 590 nm.

RESULTS

Effects of tocotrienols alone and with tamoxifen on estrogen receptor-negative human breast cancer cells. The concentration of TRF, α -tocopherol, tocotrienols and tamoxifen required to inhibit MDA-MB-435 cell proliferation by 50% was determined (Table 1). γ -Tocotrienol was a more effective inhibitor of these cells than tamoxifen. When TRF, α -tocopherol, α -, γ - or δ -tocotrienols were combined in equimolar concentration with tamoxifen, the combinations inhibited cell proliferative rate much more effectively than the compounds alone (Table 1). Figure 2 shows the inhibition of proliferation and the cytotoxic effect of a 1:1 combination of γ -tocotrienol and tamoxifen on MDA-MB-435 cells. As can be seen, most cells were viable at the IC₅₀ concentration, suggesting that the compounds are not cytotoxic. Similar results were obtained with all of the compounds and combinations tested. A synergistic effect was also observed when the combinations were tested on growth of these cells over a longer time period, as illustrated in Figure 3.

TABLE 1

Inhibition of proliferation MDA-MB-435 cells by TRF and its components, with and without tamoxifen¹

Inhibitor	IC ₅₀
	μg/mL
α-Tocopherol	>1000
TRF	180 ± 3
α-Tocotrienol	90 ± 3
γ-Tocotrienol	30 ± 2
δ-Tocotrienol	90 ± 3
Tamoxifen	90 ± 4
TRF + Tamoxifen	3.9 ± 0.2
α-Tocotrienol + Tamoxifen	1.5 ± 0.05
γ-Tocotrienol + Tamoxifen	1.9 ± 0.02
δ-Tocotrienol + Tamoxifen	5.9 ± 0.1

¹ Estrogen receptor-negative MDA-MB-435 human breast cancer cells were cultured with or without various concentrations of the test compounds. The concentration required to inhibit cell proliferation by 50% was determined, as measured by the incorporation of [³H]thymidine into DNA. The experiments were done in triplicate, and the results are averages of three experiments. Values are given as average ± SEM.

Effect of TRF, α-tocopherol and the individual tocotrienols with or without tamoxifen on estrogen receptor-positive human breast cancer cells. TRF and the tocotrienols inhibited the proliferation of MCF-7 cells more effectively than α-tocopherol, but not as effectively as tamoxifen (Table 2). The tocotrienols gave much lower IC₅₀s in MCF-7 cells than in MDA-MB-435 cells; γ- and δ-tocotrienols were the most potent. When these compounds were tested in 1:1 combination with tamoxifen, the IC₅₀s were in most cases intermediate to

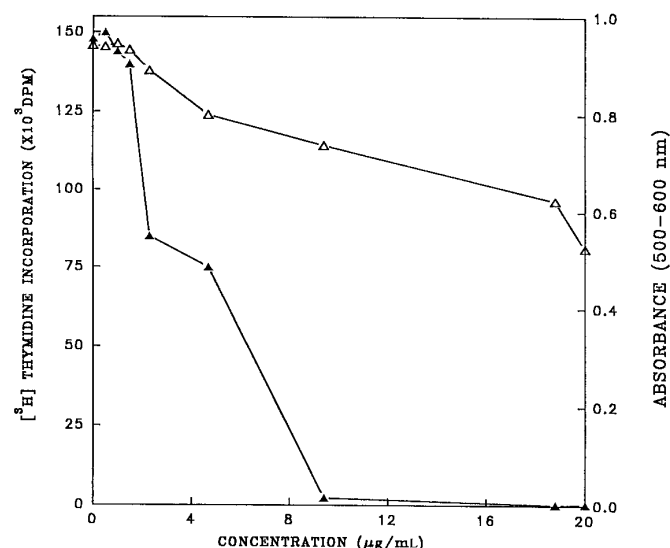


FIGURE 2 Effect of a 1:1 combination of γ-tocotrienol and tamoxifen on the proliferation (▲) and viability (△) of MDA-MB-435 cells. The cells were incubated with various concentrations of γ-tocotrienol and tamoxifen for 48 h; [³H] thymidine (18.5 μBq/well) was then added and the cells were harvested after 4 h to evaluate the incorporation of thymidine into DNA. For viability, cells were incubated with various concentrations of γ-tocotrienol and tamoxifen for 48 h, 3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide (MTT) was added (25 μL); after 3h, extraction buffer was added (100 μL) and optical density (OD) measurements made at 570 nm. Points are the average of mean values from three experiments.

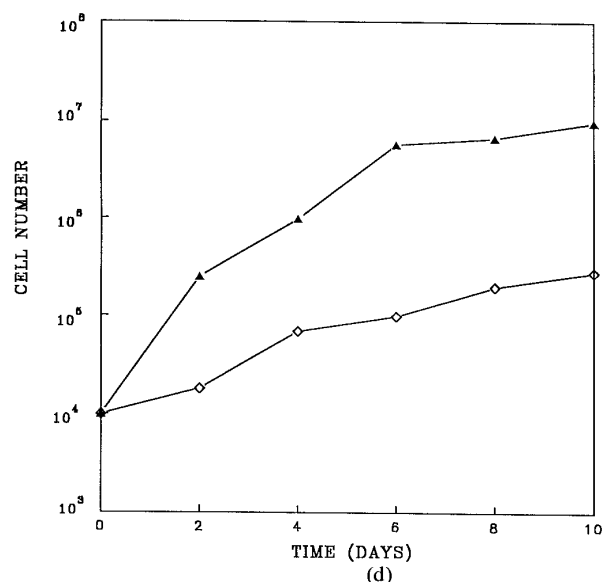


FIGURE 3 Growth of MDA-MB-435 cells in the presence (◇) or the absence (▲) of 2 μg/mL of γ-tocotrienol and tamoxifen. Cells were plated in triplicate in 60-mm culture dishes. Cells were removed by trypsinization at the specified times and were counted using a hemacytometer. Each point represents triplicate values.

those of the individual compounds (Table 2). Only γ- and δ-tocotrienols in combination with tamoxifen gave lower IC₅₀s than the compounds alone. The effects of δ-tocotrienol and tamoxifen, the most inhibitory combination, on cell proliferation and on viability, are illustrated in Figure 4. Most cells were viable at the IC₅₀ concentration. The effect of this combination on growth inhibition is shown in Figure 5.

Effects of TRF, α-tocopherol and tocotrienols on the estrogen receptor. Although MCF-7 cells are estrogen receptor-responsive, their growth is not estrogen dependent, and differences in plating density or growth medium can result in varied growth responses to estrogen (Soto and Sonnenschein 1985). To determine whether growth inhibition of MCF-7 cells by

TABLE 2

Inhibition of proliferation MCF-7 cells by TRF and its components with and without tamoxifen¹

Inhibitor	IC ₅₀
	μg/mL
α-Tocopherol	125 ± 3
TRF	4 ± 0.1
α-Tocotrienol	6 ± 0.3
γ-Tocotrienol	2 ± 0.1
δ-Tocotrienol	2 ± 0.05
Tamoxifen	0.04 ± 0.001
α-Tocopherol + Tamoxifen	46.9 ± 2
TRF + Tamoxifen	0.5 ± 0.02
α-Tocotrienol + Tamoxifen	0.1 ± 0.005
γ-Tocotrienol + Tamoxifen	0.01 ± 0.0002
δ-Tocotrienol + Tamoxifen	0.003 ± 0.0001

¹ Estrogen receptor-positive MCF-7 human breast cancer cells were cultured with or without various concentrations of the test compounds. The concentration required to inhibit cell proliferation by 50% was determined as measured by the incorporation of [³H]thymidine into DNA. The experiments were done in triplicate, and the results are averages of three experiments. Values are given as average ± SEM.

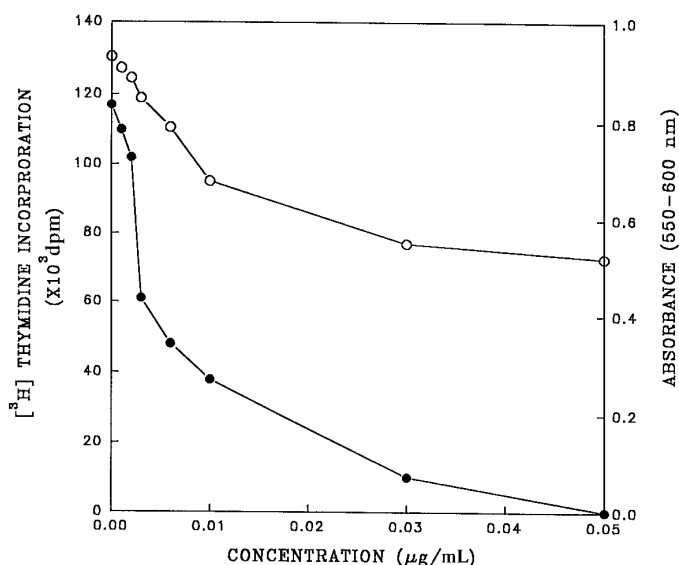


FIGURE 4 Effect of a 1:1 combination of δ -tocotrienol and tamoxifen on the proliferation (●) and viability (○) of MCF-7 cells. The cells were incubated for 5 d with various concentrations of δ -tocotrienol and tamoxifen in phenol red-free medium containing 2.5% fetal calf serum stripped of endogenous steroids. [³H] Thymidine (18.5 μ Bq/well) was then added; the cells were harvested after 4 h and the incorporation into DNA was measured. For viability, the cells were incubated as for the proliferation assay for 5 d. 3-[4,5-Dimethylthiazole]-2,5-diphenyltetrazolium bromide (MTT) was then added (25 μ L); after 3 h, extraction buffer was added (100 μ L) and optical density (OD) measurements were made at 570 nm. Points are the average of mean values from three experiments.

tocotrienols is acting through the estrogen receptor (ER) system, we treated the cells with the specified test compound at its IC₅₀ concentration and examined the ability of estradiol to reverse this inhibition, using tamoxifen as a positive control.

TABLE 3

Inhibition of proliferation of MCF-7 cells in presence or absence of estrogen

Compound	IC ₅₀ μ g/mL	[³ H]thymidine incorporation (dpm \times 10 ³ \pm SD)	
		with E ₂	without E ₂
TRF	4	18.3 \pm 2.2	19.1 \pm 2.3
α -Tocopherol	125	15.8 \pm 3.9	14.7 \pm 4.0
α -Tocotrienol	6	19.5 \pm 1.9	18.8 \pm 3.2
γ -Tocotrienol	2	21.2 \pm 3.5	20.4 \pm 2.9
δ -Tocotrienol	2	20.9 \pm 4.6	20.5 \pm 3.3
Tamoxifen	0.04	44.5 \pm 2.1	20.9 \pm 2.8

¹ Estrogen receptor-positive human breast cancer cells were cultured with the test compounds at their IC₅₀ concentration in the presence or absence of 100 nM 17- β Estradiol (E₂), and the incorporation of [³H]thymidine into DNA was measured.

Table 3 shows that their inhibitory effect was not reversed by the addition of excess estradiol as occurs with tamoxifen. This indicates that their antiproliferative activity is probably not mediated through the ER.

DISCUSSION

Cell culture and animal studies have shown that tocotrienols, the unsaturated homologues of tocopherols, have anti-cancer activity. We previously reported that tocotrienols inhibit MDA-MB-435 cells, whereas α -tocopherol is ineffective. In the present study, we have shown that tocotrienols are effective inhibitors of both ER-negative and -positive cells and that combinations with tamoxifen produce a synergistic effect.

Tamoxifen, a nonsteroidal estrogen antagonist, is effective in arresting the growth of estrogen-responsive cells in a growing tumor and thus is widely used in the treatment of hormone-responsive tumors (Furr and Jordan 1984). However, most breast cancers are heterogeneous and consist of hormone-responsive as well as nonresponsive cells (Tiwari et al. 1991). A treatment regimen using a negative growth inhibitor coupled with an antihormonal drug would effectively target both types of tumor cells. Our results indicate that tocotrienols and tamoxifen have mutually potentiating effects on MDA-MB-435 cells and, in some cases, on MCF-7 cells, as measured by their effect on [³H] thymidine incorporation.

Even though tamoxifen is the most prescribed drug for breast cancer treatment, its uses are limited. Tumors invariably develop resistance which may be caused by the intrinsic estrogen agonist properties of tamoxifen (Osborne et al. 1995). Also, concerns have been raised that this drug may increase the incidence of new primary malignancies, such as endometrial, liver and colorectal cancers (Rutqvist et al. 1995). Because tocotrienols inhibit both ER⁺ and ER⁻ cells, their potential for breast cancer treatment is of interest. The positive synergism observed with these compounds may allow the use of lower doses of tamoxifen, thus reducing the risk of side effects for patients.

The precise mechanism of action of tocotrienols is at present unknown, but the inhibition of cells might be related to their antioxidant properties (Serbinova et al. 1991). In smooth muscle cells, α -tocopherol has been shown to inhibit protein kinase C (Chatelaine et al. 1993), which has been implicated in signal transduction and cellular proliferation (Nakamura and Nishizuka 1994). It is possible that tocotrienols might

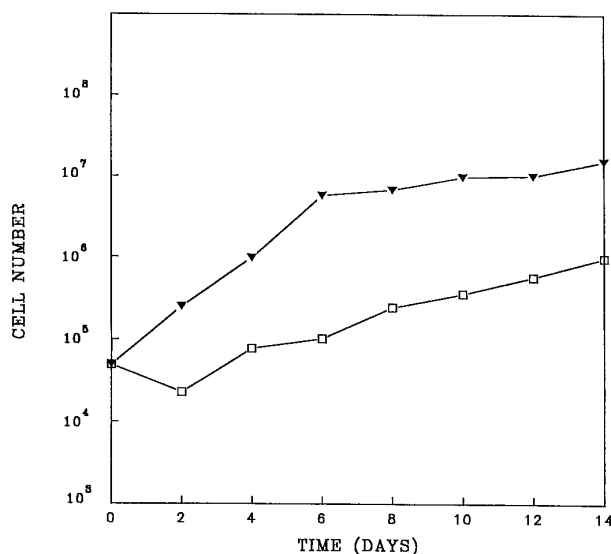


FIGURE 5 Growth of MCF-7 cells in presence (□) or absence (▼) of 0.003 μ g/mL of 1:1 combinations of δ -tocotrienol and tamoxifen. Cells were plated in triplicate in 60-mm culture dishes, removed by trypsinization at the specified times and counted using a hemocytometer. Each point represents triplicate values.

exert their antiproliferative properties by interfering with signal transduction events involving protein kinase C. The synergism between tocotrienols and tamoxifen indicates that they are acting by different mechanisms. Our data suggest that tocotrienols act via an ER-independent pathway. However, this does not rule out the involvement of the ER. Previous studies have shown that increased phosphorylation of the estrogen and progesterone receptors can alter their activity (Denner et al. 1990). Therefore, tocotrienols might exert their effect in part by interfering with the phosphorylation state.

In conclusion, our results show that tocotrienols inhibit proliferation of both MDA-MB-435 and MCF-7 cells. Synergistic effects with tamoxifen were observed in the MDA-MB-435 cells but only γ - and δ -tocotrienols and tamoxifen were synergistic in MCF-7 cells.

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